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The effect of gibberellic acid and the retardant AMO-1618 on the initial stages of growth and nucleic acid synthesis in etiolated soybean seedlings.

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THE EFFECT OF GIBBERELLIC ACID AND THE RETARDANT AMO-1618 ON THE INITIAL STAGES OF GROWTH AND NUCLEIC ACID SYNTHESIS IN ETIOLATED SOYBEAN SEEDLINGS

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ABSTRACT

The plant growth retardant AMO-1618 is known to block the synthesis of gibberellins in various plant systems. The resulting retardation of the plant can sometimes be reversed in a kinetic manner by the application of gibberellin. In other systems, and/or at other concentrations, retardants have been shown to promote growth. It has been suggested that such retardant effects are not associated with gibberellin action but are a separate physiological effect. The present study demonstrates that such a promotive effect of AMO-1618 in roots and hypocotyls of etiolated soybean seedlings may indeed be mediated by gibberellin application. Evidence is presented that this mediation involves changes in growth rates and in nucleic acid synthesis. The possible kinetics of this interaction are discussed.

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ABBREVIATIONS

Alar	N-dimethylaminosuccinamicacid
AMO-1618	2'-isopropyl-4'-(trimethylammoniumchloride)-5'-methylphenylpiperidinecarboxylate
CCC	β-chloroethyltrimethylammoniumchloride
DNA	deoxyribonucleicacid
2,4-D	2,4-dichlorophenoxyaceticacid
GA	gibberellic acid
IPA	N^6-(Δ^2-isopentanyladenosine)
MAK	methylated albumin on kieselguhr

Nucleic acid fractions

4S	transfer RNA
5S	soluble ribosomal RNA subunit
DNA	DNA and associated RNA
lRNA	light ribosomal RNA subunit (18S)
hRNA	heavy ribosomal RNA subunit (25S)
mRNA	messenger RNA
TB-RNA	tightly bound RNA

O.D.	optical density unit
^{32}Pi	inorganic phosphate with radioactive ^{32}phosphorus
Phosphon	tributyl-2,4-dichlorobenzylphosphoniumchloride
RNA	ribonucleic acid
S	Svedberg unit
Tris-HCl	Tris (hydroxymethyl) aminomethane adjusted to pH with hydrochloric acid
U.V.	ultraviolet light

INTRODUCTION

Work in this and many other laboratories has attempted to correlate the actions of AMO-1618 and GA. Other work has been directed toward uncovering specific sites or modes of action of each of these chemicals. It now seems fairly clear that at least one of the actions of AMO-1618 is to block a specific step in the biosynthesis of GA. Thus AMO-1618 should have demonstrable effects on any plant processes that require de novo production of GA for their completion and these effects should be completely reversed by externally supplied GA. Previous work in this laboratory (Yu Tu, 1970) showed that general changes in total cell wall, protein, and nucleic acid content of pea tissues simply reflect the overall growth responses of the plant to these chemicals. Since their effects are broad it seems that the major action of GA is at the level of nucleic acid synthesis.

The purpose of this study was to determine the specific effects of these two chemicals at the level of nucleic acid metabolism in soybean seedlings. Initially it was necessary to establich reliable fractionation procedures for nucleic acids using MAK columns. Finally, an attempt was made to demonstrate a kinetic interaction between the growth retardant AMO-1618 and the plant growth regulator GA with a special view to determining whether or not growth responses to these chemicals are reflected in some or all nucleic acid fractions. Details of these two efforts are presented in this thesis.

REVIEW OF LITERATURE

In the recent review of Gibberellins by Lang (1970) evidence was cited with respect to their distribution, biosynthesis, and mode of interaction with other plant hormones and some retardants. It appears that gibberellins are ubiquitous in distribution (Paleg, 1965; Lang, 1970; Tamura et al., 1969) and have multiple forms (Lang, 1970). Each different species appears to contain its own set of gibberellins and within a single species the kinds and quantities of the different gibberellins varies with organ and developmental stage.

Jones and Phillips (1966) have demonstrated that gibberellins are synthesized in light-grown sunflowers, in the young leaves of the apical bud, and in the apical (3-4 mm) root tips. Developing seeds have also been shown to be sites of gibberellin synthesis (Kende and Lang, 1964). As a result, some mature seeds such as peas contain stores of gibberellins in a "bound" form (Bardense et al., 1968) making them independent of endogenous production for the first 2-3 days of germination. The same group showed that Pharbitis seeds contain little stored hormone and require immediate synthesis of gibberellins.

Dwarf and tall varieties of the same species are common material used in attempts to elucidate the mode of action of gibberellin. Using dwarf and tall varieties of peas, Kende and Lang (1964) have shown that the activity of

some gibberellins can be inhibited by light while that of others cannot. They have also shown that etiolated dwarf peas, which grow tall, have a gibberellin level equal to that of light-grown dwarf peas, which are dwarfed. Radley (1970) has shown that, in light-grown wheat, GA stimulates the growth of tall cultivars but not the dwarfs (the reverse of the effect in peas), and in addition, that the dwarf cultivars contain much higher GA levels than do tall cultivars. She suggested that in the dwarf there might be a "block to the utilization of GA caus(ing) an accumulation of the hormone".

In 1970 Lang outlined the biogenesis of gibberellins, as it is presently understood, from acetyl-CoA and mevalonate. It is interesting to note that two other plant hormones can be formed from intermediates in the same pathway; namely, the cytokinin IPA from isopentenyl pyrophosphate and abscisic acid from farnesyl pyrophosphate.

It is also clear that the retardants AMO-1618, CCC, and phosphon act as inhibitors of gibberellin synthesis by blocking the conversion of trans-geranylgeranyl pyrophosphate to copalyl pyrophosphate (Dennis et al., 1965). As a result of this effect Lang (1970) was prompted to say that "these retardants can thus serve as a very useful tool in studying certain questions of gibberellin physiology. They are a chemical knife that can do for us what the decapitation of the Avena coleoptile and other plants did in the study of auxin physiology". He cautioned that these retardants

probably have other effects as well and that an interaction between GA and a retardant "can be related causally only if the effect on growth can be completely overcome by applied GA". As one example he cites evidence of CCC promoting growth in low concentrations possibly by becoming an additional source of reduced nitrogen for growth.

The retardant AMO-1618 which is used in this work was first reported by Wirwille and Mitchell (1950), and the interrelation between its molecular structure and function was characterized by Cathey (1965). Kende and Lang (1964) showed that AMO-1618 could dramatically inhibit the biosynthesis of gibberellin in developing pea seeds (by almost 90%) at concentrations greater than 10^{-4}M, while effectively reducing the growth of the seeds in fresh weight. However a lower concentration of approx. 10^{-5}M reduced endogenous GA levels by 60% without significantly affecting growth. They concluded that this result at the lower concentration "elimina(ted) the possibility that the changes in the gibberellin content of the seeds are the consequence rather than the cause of the changes in growth".

Halevy and Cathey studied the effect of gibberellins (1960a), of AMO-1618 (1960b), and of a combination of them in high concentration (Halevy, 1963) on the growth of cucumber seedling roots and hypocotyls. They found that the seedlings responded differently to the various gibberellins and that differing concentrations resulted in promotive and retarding effects on both organs. Their data concerning the

effect of various concentrations of AMO-1618 shows that a concentration less than 10^{-5}M stimulates growth of dark-grown roots and hypocotyls while greater concentrations progressively retard both. Halevy (1963) showed an inverse relationship between catalase and growth resulting from applications of GA and AMO-1618. A concentration of GA which stimulated hypocotyl growth but not radicle growth caused catalase levels to fall in hypocotyls but not in radicles. A concentration of AMO-1618 which inhibited both hypocotyl and root growth stimulated catalase in each. Other retardants such as CCC and Alar were found not to affect catalase in this way.

Paleg (1965) has indicated that enhancement of the release of hydrolytic enzymes from aleurone layers of cereal grains by gibberellins was first demonstrated in work done independently by Yomo (1958, 1960) and Paleg (1960). From subsequent evidence from other tissues he is led, in his review, to the conclusion "that the hormonal mechanism results in a mode of action in which the synthesis of enzymically active protein occurs".

Much effort has been expended, therefore, in an attempt to find a mode of action of gibberellin and other hormones at the transcriptional and/or translational level. For example, Johri and Varner (1968) have shown that "nuclei isolated from shoots of light-grown peas in the presence of gibberellic acid (10^{-8}M) show a higher rate of DNA-dependent RNA synthesis than the untreated nuclei" and that this RNA is

qualitatively different from the control. Fractionations on MAK columns showed the increased activity to be in the DNA-RNA and in the TB-RNA fractions. They suggest that "enhanced RNA synthesis could be due to increase in template sites on DNA and/or due to increase in the activity of the RNA polymerase enzyme itself".

Since "the nuclei responded to the hormone only if they were isolated in its presence" they have suggested that "it is conceivable that some factor(s) present in the cytoplasm or nucleus is involved before GA elicits the final response. The failure of purified nuclei to respond to hormone is perhaps related to the loss of this factor during isolation. Alternatively the hormone may in some way be involved in transporting a factor into the nucleus". Recently Matthyse and Phillips (1969) have shown that a protein mediator is involved in the auxin (2,4-D)-induced enhancement of DNA dependent RNA synthesis by isolated nuclei from tobacco or soybean tissue cultures or chromatin from pea buds. Yasuda and Yamada (1970) have recently shown that 2,4-D complexes with histones during callus induction (dedifferentiation) in pea epicotyls.

On the other hand, Gayler and Glasziou (1969) have shown in sugar cane internodes that GA, at a high concentration ($5x10^{-3}M$), has no effect on peroxidase nor does it affect invertase breakdown or synthesis, but it does "increase the enzyme forming capacity" for invertase. Thus, they suggest that GA and auxin (from other work in the same

paper) may act by stabilizing specific mRNA species thereby increasing their "enzyme-forming capacity". It should be noted that Johri and Varner (1968) used a physiological concentration of gibberellin while Gayler and Glasziou used a very high concentration thus putting into question the physiological significance of their results.

MATERIALS AND METHODS

1. Growth of Soybean Seedlings

All experiments were conducted using soybeans, Glycine maximus L. var. Harrowsoy 63. The seeds were graciously supplied by the Research Station, Canadian Department of Agriculture, Harrow, Ontario. Seeds were surface sterilized for 20 minutes prior to use with javex-water*(1:16^{V}/v) and grown in the dark in high humidity for various periods of time up to 6 days. Younger seedlings (up to 3 days old) were grown at 30°C in sterile plastic petri dishes containing initially 20 ml of test solution or water as the control. At the end of every 24 hour period, each dish of seeds was rinsed and supplied with 5 ml of fresh solution. Older seedlings were either grown at 30°C in vermiculite in plastic trays or at 24°C between glass plates (single diamond, 20x20 cm) and filter paper moistened with appropriate solutions. In this procedure surface sterilized seeds were first imbibed in sterile petri dishes in the test solution overnight and seeds with a healthy appearance were set in a line 3 cm from the top of the glass plate under the moistened paper and supported in place by an elastic band which ensured that the paper and seeds remained in place when the plates were placed upright in a metal rack. The lowest 2 cm of each plate was enclosed in a small reservoir made from

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*Javex contains 5.25% NaOCl as the active agent.

folded aluminum foil. The rack, with its several plates, was placed in a cardboard box, lined with saran wrap, and covered with aluminum foil to ensure darkness and a high humidity.

2. Incubation of Tissue

In each case the tissue was held in a solution containing 10^{-4}M ammonium citrate, 1% sucrose, and 50µg/ml chloramphenicol with or without GA or AMO-1618, in varying concentrations and for appropriate times. Hypocotyls and roots were divided into approximate 2 cm sections while cotyledons were sliced into 3 sections along the long axis with a razor blade.

3. Incorporation of ^{32}Pi

For isotope incorporation studies either 0.5 or 1.0 mC of ^{32}Pi (carrier free orthophosphate) was added to each flask usually containing 30 ml of incubation medium and tissue. The contents were continuously agitated in a water bath at 30°C for 2 or 3 hours, respectively. Incubation was halted by washing the tissue three times with ice-cold 0.05 M phosphate buffer (pH 6.7) then ice-cold distilled water. The tissue was then lyophilized in a freeze-drier for extraction of nucleic acids.

4. Extraction and Purification of Nucleic Acids

Extraction was done essentially by the Phenol-Tris buffer method employed by Cherry (1966). Samples were

homogenized at 5°C in a solution containing 40 ml 0.01 M Tris-HCl (pH 7.6), 0.06 M KCl, 0.01 M $MgCl_2$; 4.6 ml 11% duponol; 65 ml phenol (preequilibrated with the above Tris, KCl, $MgCl_2$ solution). All extractions were carried out at 0°C or over ice. Homogenates were reextracted twice with phenol and bentonite. The aqueous supernatant was made 0.2M with potassium acetate and the nucleic acids precipitated with 2 volumes of ethanol for at least 2 hours at -10°C. The precipitate was dissolved in 5-10 ml of 0.05 M sodium phosphate buffer (pH 6.7) and dialyzed overnight against cold 0.05 M buffer.

5. Chromatography on MAK Columns

Purified nucleic acid extracts were fractionated on columns of Methylated Albumin on Kieselguhr essentially according to the method of Osawa and Sibatani (1967). This method utilizes a three-layered column of cellulose powder, MAK, and kieselguhr instead of the original four-layered column of Mandell and Hershey (1960). (See Appendix).

Typically, 35 O.D.'s of the partially purified plant nucleic acid were dissolved in 40 ml of starting buffer, added to the column, and eluted with a linear gradient of NaCl from 0.3 M to 1.5 M in 0.05 M phosphate buffer (pH 6.7) using 250 ml of each. Fractions containing 5-6 ml were collected and the ultra-violet absorbancy of each fraction was determined at 258 mμ using either a Unicam Spectrophotometer (model Sp 800) or a Beckman DB-G grating spectrophotometer.

The glass columns used were designed and constructed in this laboratory from simple glass tubes of 2.5 cm ID and 24 cm length to a coarse sintered glass disc. The lower end was tapered to fit thin tubing which could be easily clamped.

6. Scintillation Counting

Detection of ^{32}Pi was tried using either

a) toluene, PPO, and POPOP

or b) toluene, PPO, POPOP, and methyl cellosolve (1000 ml, 4 g, 50 mg, and 700 ml, respectively) (Warner and McClean, 1968)

or c) by detection of "Cerenkov radiation" directly from the aqueous solutions eluted from the column (Haviland and Bieber, 1970).

The Nuclear-Chicago scintillation system (Model 6850) was used in all cases.

For the "Cerenkov" method 5 ml of each fraction was mixed with 5 ml of distilled water in glass scintillation vials and the radiation counted at the balance point.

Where specific activities of nucleic acid extracts and fractions from various treatments were compared the number of counts obtained for each calculation was corrected for decay.

FIGURE 1

Fractionation on MAK Column of 6 day old Soybean Nucleic Acids

A typical profile of a MAK fractionation of soybean nucleic acids using the simplified column of Osawa and Sibatani. The nucleic acids for this example were extracted from 6 day old hypocotyls. Peaks I to VI refer to 4S, 5S, DNA, lRNA, hRNA, and mRNA, respectively. The solid line is the U.V. profile while the dotted line represents the radioactivity.

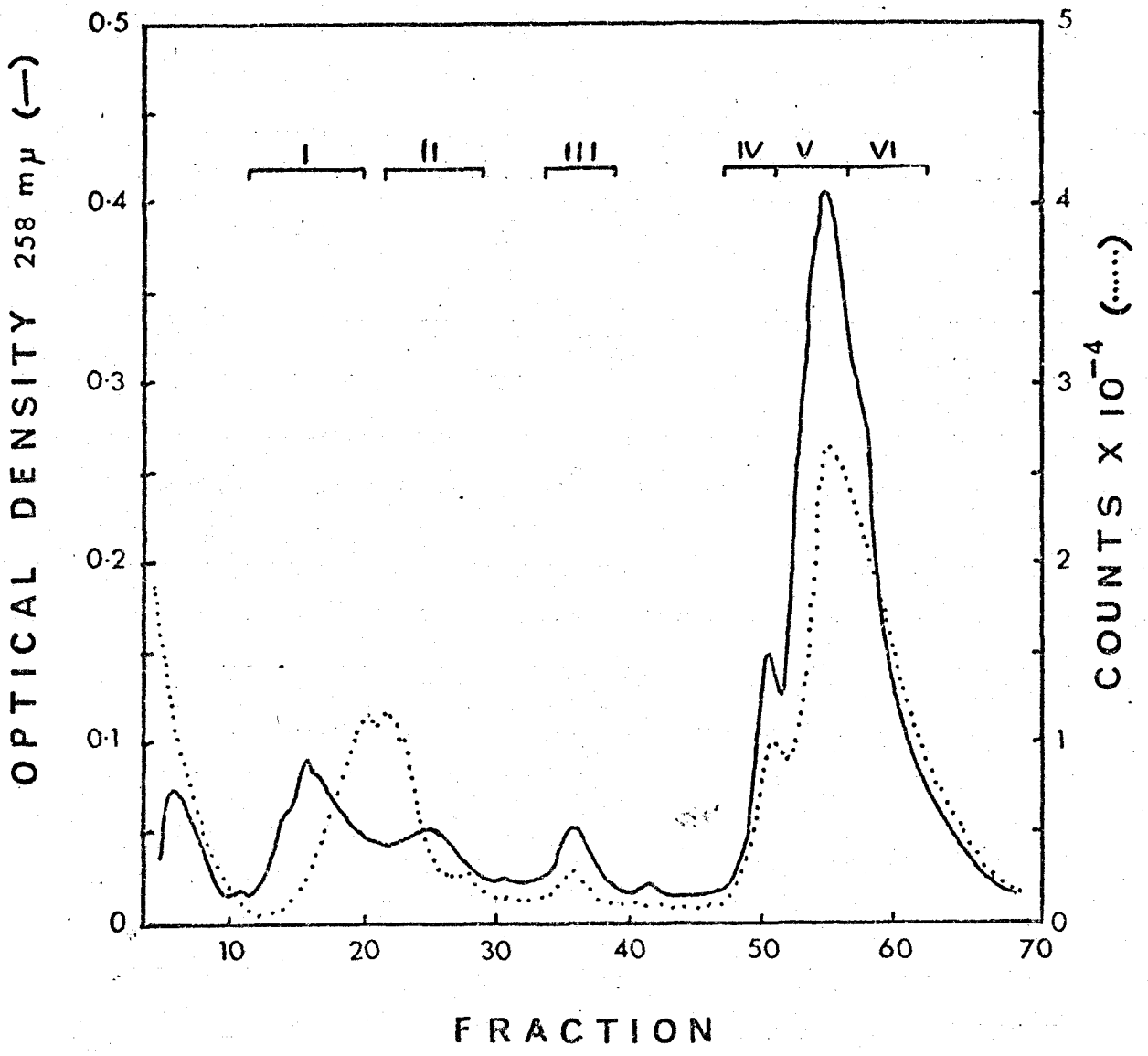

Fig. 1. Fractionation on MAK Column of 6 day old Soybean Nucleic Acids

RESULTS

I Standardization of Procedures

a) MAK column

The original procedures of Mandell and Hershey (1960) have undergone a wide variety of alterations at the hands of different workers. Attempts have been made to further elucidate the mode of action of the MAK in separating the various species of nucleic acid. It is still not clear exactly how the column effects the fractionation except that factors such as molecular size, hydrogen bonding content, and base composition seem to contribute to the separation. More recently Johri and Varner (1970) have implicated secondary structure in the separation of "mRNA" and TB-RNA.

Other workers have attempted to determine the limits of flexibility of the column. Attempts were made to utilize some of the simplified procedures in the fractionation of plant nucleic acids. The three-layered column of Osawa and Sibatani (1967) gives very reliable and repeatable fractionations whose U. V. and radioactivity profiles compare quite favourably with previous work using the classical Mandell and Hershey procedures (Fig 1). It was found, as have others, that the column could be safely reused, but in all the work reported here each column was used for only one fractionation. In addition

TABLE 1

Comparison of Cerenkov and scintillation counting of ^{32}Pi incorporated into nucleic acids.

Counting procedure	Counts/min/ml of nucleic acid extract		
	Sample no.		
	1	2	3
Scintillation	37,870	15,370	5,940
Cerenkov	7,310	3,110	970
Cerenkov as % of Scintillation	19	20	16

Aliquots (0.1 ml) from each of 3 different nucleic acid fractions were counted as outlined in the Materials and Methods for each counting procedure.

it appears that storing the column for several days at 4°C has no effect on the operation of the column although the columns used in this work were stored in this way for no longer than three days. As well, the variation in glass column used seems to have had no ill effect on performance.

b) Scintillation counting

Haviland and Bieber (1970) have recently demonstrated that Cerenkov counting may well be "the method of choice for the detection of $^{32}P_i$". In this lab this method has not only given comparable and repeatable results but has the added advantages, as cited by Haviland and Bieber, of not requiring added scintillator as well as allowing the complete recovery of sample after counting. All this, along with the maintenance of relative efficiency, certainly has made it the "method of choice" in this work.

Table 1 shows a comparison of Cerenkov counting with that using scintillation fluid (Warner and McClean,1968). 0.1 ml of each of three different samples of radioactive nucleic acid extract was counted for one minute by each of the two methods. It can be seen that even at these low count levels the Cerenkov counting gave relative results quite comparable to the scintillation counting method.

TABLE 2

Distribution of $^{32}P_i$ after incorporation into nucleic acids of water-grown soybean seeds and seedlings.

organ	age	Radioactivity as total counts/min x 10^{-3} (% of total added to column) added to column	lost in wash	first 10 tubes	Nucleic Acid	not recovered	
seed	12 hr	759	-	141 (19)	6.2 (0.82)	612 (81)	a
seed	24 hr	689	-	147 (21)	8.3 (1.2)	534 (78)	a
seed	48 hr	51	-	18 (35)	1.8 (3.5)	31 (60)	b
hypocotyl	6 days	136	45 (33)	18 (13)	34 (25)	38 (28)	b
root	6 days	976	103 (11)	40[c] (4)	594 (61)	239 (24)	b

[a]Scintillation counting

[b]Cerenkov counting

[c]Estimated from incomplete data

Incorporation, extraction, and MAK chromatography were performed as outlined in the Materials and Methods. Age refers to time after first app,ication of distilled water which was used throughout.

II The Interaction of GA and AMO-1618

a) Initiation of Incorporation of ^{32}Pi into Nucleic Acid Fractions and Distribution of Radioactivity

In order to estimate the onset of a level of nucleic acid synthesis by imbibing seeds, surface sterilized seeds were placed in sterile petri dishes in the dark at 30°C and allowed to imbibe water for 12, 24, and 48 hours before incubation with 1.0 mC ^{32}Pi for 2 hours. Table 2 shows the general distribution of radioactivity after elution through the column. Clearly the molecules into which the ^{32}Pi are first incorporated consist mainly of those species which either remain bound to the column after elution or are lost in the column wash during loading of the column. Obvious incorporation into nucleic acid fractions occurs sometime between 24 and 48 hours after imbibition starts (acceptable profiles have been achieved after 36 hours of imbibition). At this stage not only has incorporation occurred into all the nucleic acid fractions but an increase in incorporation into those molecules which elute first from the column has also occurred. This is all at the expense of the molecules which either remain bound to the column after elution or are lost in the pre-wash.

Table 2 also shows the distribution of radioactivity incorporated into fully growing organs. For 6 day old hypocotyls the radioactivity lost in the pre-wash plus that left bound to the column equal 61% of the total added to the column (similar to the 48 hour seed). On the other

TABLE 3

The onset of incorporation of ^{32}Pi into nucleic acid fractions from MAK chromatography.

water imbibition (hr)	Specific activity (cts/OD, 258 mu)						
	4S	5S	DNA	lRNA	hRNA	mRNA	average, all peaks
4	120	150	120	35	22	71	86
12	120	93	110	36	23	37	70
24	150	190	64	35	21	51	85
48	3400	4300	2700	1700	840	900	2300

Incorporation, extraction, and MAK chromatography were performed as outlined in the Materials and Methods. Age refers to time after first application of distilled water which was used throughout.

hand, incorporation into nucleic acids has greatly increased, compared to 48 hour seeds, almost totally at the expense of the activity that had eluted in the first ten, preprofile tubes.

Roots show a great increase in incorporation into nucleic acids predominantly at the expense of the precursors in the wash . In general it appears that the radioactivity bound to the column after elution remains as a uniform percentage of total incorporation and any increase in incorporation into nucleic acids seems to arise out of utilization of "precursors" that may comprise a great part of the activity eluted before the profile. Much work has been expended by various workers in attempting to elucidate the nature of the TB-RNA left on the column. It would seem from the data presented here that an investigation of the radioactive compounds that elute prior to the nucleic acid profile might be quite useful.

Table 3 shows that the average specific activity of peak tubes of nucleic acids extracted from seeds up to one day old is fairly uniform and that they are only 3.5% as active in incorporation of ^{32}Pi as are two day old seeds. This low level of incorporation at early imbibition stages could either represent a minimal level of nucleic acid synthesis occurring in the seeds or could represent that level of activity contributed by contaminating organisms not inhibited by the chloramphenicol in the incubation medium. If the latter is the case it is important to re-

TABLE 4

The effect of high concentrations of AMO-1618 on the incorporation of ^{32}Pi into nucleic acid fractions of imbibing seeds.

AMO-1618	Specific activity as % of control						
	4S	5S	DNA	lRNA	hRNA	mRNA	Ave.
$3x10^{-4}M$	77	92	92	85	93	96	88
$3x10^{-4}M$	35	21	-	15	6.5	7.9	19

Seeds were imbibed for two days in either distilled water or the appropriate AMO-1618 solution before ^{32}Pi incorporation. All procedures were done as outlined in Materials and Methods.

cognize that the contribution of contaminating organisms to the specific activity of ^{32}Pi incorporation into nucleic acid fractions is no greater than 3.5% of the total.

b) The Effect of High Concentrations of AMO-1618 on the Incorporation of ^{32}Pi into the Nucleic Acids of Imbibing Seeds.

A concentration of $3x10^{-3}M$ AMO-1618 is lethal to imbibing seeds and allows only limited growth of the root when grown for more than 48 hours. Table 4 shows that this effect is reflected in a much reduced incorporation of ^{32}Pi into all fractions of nucleic acids. It is especially interesting, given the importance of DNA in the production of RNA, that the incorporation into the DNA-RNA is reduced to background levels.

On the other hand AMO-1618 at $3x10^{-4}M$ retards the development of both roots and hypocotyls but is not lethal to the seedlings. This is also reflected in an average 11% reduction in specific activity.

c) The Effect of GA and AMO-1618 on Cotyledon Nucleic Acids.

Since cotyledons perform mainly a storage function and exhibit little or no cell division in their development it was felt that they would represent a tissue which would offer the least number of physiological variables and thus would be the most appropriate tissue with which to compare the activities of these compounds. Variables were further

TABLE 5

The effect of GA and retarding AMO-1618 on incorporation of ^{32}Pi into nucleic acid fractions of cotyledons.

Pretreatment[a]	Specific activity cts/OD 258 mu						
	4S	5S	DNA	lRNA	hRNA	mRNA	AVE[b]
AMO 12hr 30°C	2220	3960	9470	1110	1330	1320	3310
AMO 12hr 25°C	--	--	1060	350	450	640	550
GA 12hr 25°C	--	--	1250	550	630	1030	860
AMO + GA 12hr 25°C	1230	1130	1190	460	640	1050	830
AMO 1 hr 25°C	770	2060	1700	580	650	860	950
GA 1 hr 25°C	2410	3010	4990	820	1010	1280	2020
AMO + GA 1 hr 25°C	1580	2140	2830	530	660	760	1200

[a] all concentrations = $3x10^{-4}$M.

[b] average of last 4 peaks only.

Cotyledons from six day old water-grown soybean seedlings were pretreated as indicated before ^{32}Pi incorporation and subsequent extraction and MAK fractionation as described in the Materials and Methods.

reduced by the use of dark-grown cotyledons thus minimizing, in addition, chloroplast activity.

Table 5 shows the effects of sub-lethal ($3x10^{-4}M$) doses of AMO-1618 either separate from GA or in combination with it, on the incorporation of ^{32}Pi into the nucleic acid fractions of 6 day old, water-grown cotyledons. It can be seen from the average specific activity that incorporation into GA treated cotyledons is always the greatest and into AMO-1618 the least with the combination of AMO-1618 and GA (equimolar) having an intermediate activity. It is significant as well, that the responses are different with short and long pretreatment. GA caused 50-100% greater ^{32}Pi incorporation in both 1 and 12 hour pretreatments than did AMO-1618 while the mixture of AMO-1618 and GA acted like AMO-1618 in the first hour but like GA after 12 hours. Does this imply that AMO-1618 acts quickly, and that GA is able to overcome its affect on nucleic acid fractions only after a time? It must be remembered that this is the response of this tissue to these chemicals in concentrations at which each separately is retarding to the plant.

d) <u>The Effect of GA and AMO-1618 on the Growth of Soybean Seedlings</u>

Gibberellic acid (GA) is generally known as a stimulator of plant growth while AMO-1618 is known as a retardant. In some plants GA can be shown to overcome the action of AMO-1618 in a competative way but in others the interrelationship is not as clear. It is important, then,

FIGURE 2

The Effect of GA and AMO-1618 on the Growth of Roots of 6 day old Soybean Seedlings

and

FIGURE 3

The Effect of GA and AMO-1618 on the Growth of Hypocotyls of 6 day old Soybean Seedlings

Soybean seedlings were grown for 6 days on glass plates as outlined in the Materials and Methods in various concentrations of AMO-1618 and GA separately or in combination. Ten of the most typical seedlings from each treatment were selected and the average length of their roots and hypocotyls was determined.

(_____) AMO-1618
(- - -) GA
(.....) 10^{-5}M AMO-1618 + varying concentrations of GA
(-.-.-) 10^{-4}M AMO-1618 + varying concentrations of GA

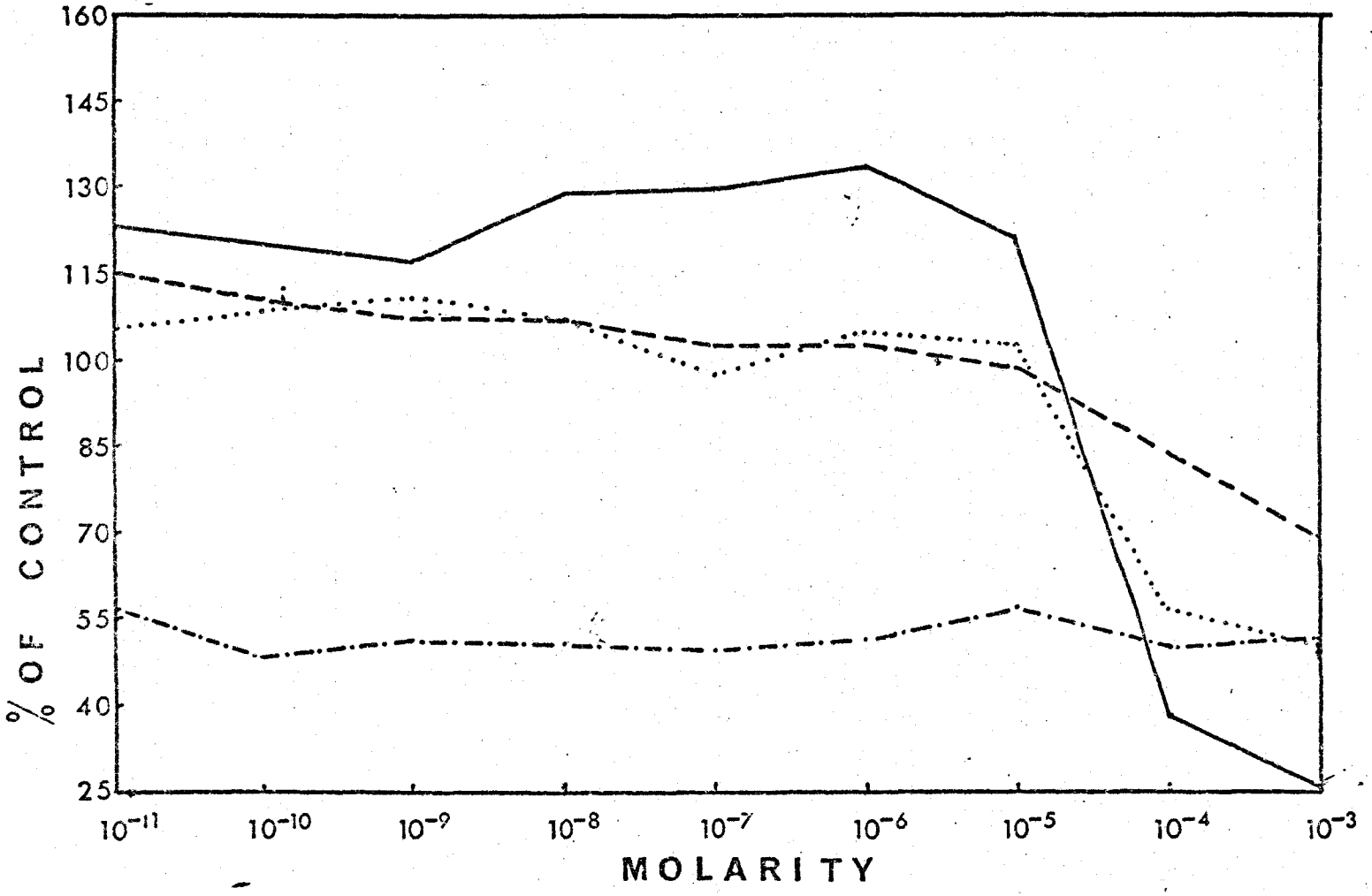

Fig. 2. The Effect of GA and AMO-1618 on the Growth of Roots of 6 day old Soybean Seedlings

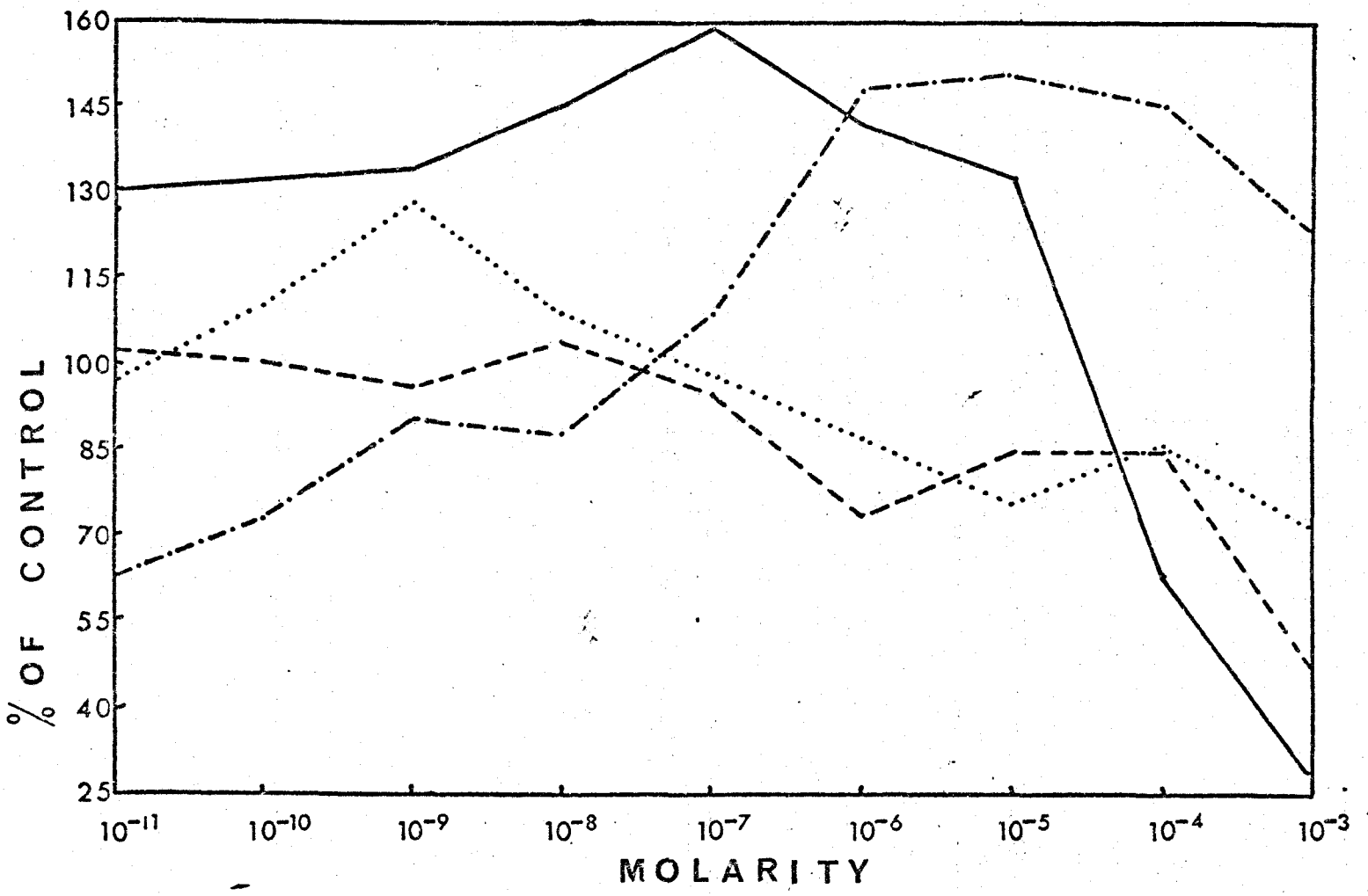

Fig. 3. The Effect of GA and AMO-1618 on the Growth of Hypocotyls of 6 day old Soybean Seedlings

to determine the mode of interaction between these two chemicals on the overall growth of the Harosoy 63 variety of soybeans chosen for this work.

The results of a typical experiment are shown in Fig. 2 and Fig. 3 in which surface sterilized seeds were grown in the dark for 6 days between glass plates and filter paper soaked with the appropriate concentrations of test solutions. For roots it can be seen that GA is only slightly stimulatory to growth up to a concentration of 10^{-5}M above which it begins to inhibit growth. AMO-1618, on the other hand, is much more effective in stimulating growth, again up to a concentration of 10^{-5}M above which it becomes much more retarding than GA. GA appears to be able to completely overcome the promotive effects of AMO-1618 at 10^{-5}M but it is unable to overcome the retarding effects of AMO-1618 at 10^{-4}M. It appears, then, that there is a direct relationship in soybean roots between GA and AMO-1618 when AMO-1618 is used in a promotive concentration but not when AMO-1618 is in a retarding concentration.

For hypocotyls the interaction seems somewhat more complicated. For roots, the "critical concentration" at which promotion turns into retardation was the same for both GA and AMO-1618; namely, between 10^{-5}M and 10^{-4}M. But for hypocotyls this "critical concentration" is different for GA being between 10^{-8}M and 10^{-7}M.. This concentration appears to be significant as well in the interaction of AMO-1618 and GA since at lower concentrations than this of GA a

TABLE 6

The effect of GA and AMO-1618 on the incorporation of ^{32}Pi into the nucleic acids of soybean roots and hypocotyls.

	% of control				
	Specific Activity				
Test	Ave. of 4S & 5S	DNA	Ave. of 1-, h-, & mRNA	Ave. of all peaks	Length
Roots					
GA $10^{-6}M$	9	16	33	19	103
GA $10^{-3}M$	19	5	22	15	70
AMO $10^{-7}M$	177	28	82	96	130
AMO $10^{-4}M$	192	68	140	133	37
GA $10^{-8}M$ AMO $10^{-4}M$	45	20	70	45	51
GA $10^{-4}M$ AMO $10^{-4}M$	46	10	24	27	51
Hypocotyls					
GA $10^{-6}M$	205	262	295	254	74
GA $10^{-3}M$	35	54	59	50	48
AMO $10^{-7}M$	261	317	395	324	159
AMO $10^{-4}M$	42	83	44	56	64
GA $10^{-8}M$ AMO $10^{-4}M$	69	68	83	73	88
GA $10^{-4}M$ AMO $10^{-4}M$	267	226	236	243	145

The roots and hypocotyls were harvested from the seedlings used in the growth experiment (section II (d) of these results). All procedures were as outlined in Materials and Methods. The % of control lengths are from the growth experiment.

promotive concentration of AMO-1618 (10^{-5}M) has control while at higher GA concentrations the GA seems to have control.

The interaction of GA with a retarding concentration of AMO-1618 (10^{-4}M) is quite different except that the critical GA concentration is again between 10^{-8}M and 10^{-7}M. Increasing concentrations of GA from 10^{-11}M to 10^{-7}M seem to progressively overcome the retarding effect of AMO-1618 at 10^{-4}M; but at higher concentrations of GA rather than the GA maintaining control the GA and AMO-1618 seem to act synergistically, promoting growth considerably where each separately would retard growth. It is quite clear then, though not surprising, that the mode of interaction between these two chemicals is somewhat different in the two actively growing parts of the seedlings: the roots and the hypocotyls.

e) The Effect of GA and AMO-1618 on the Incorporation of ^{32}Pi into the Nucleic Acids of the Growing Parts of Soybean Seedlings

Table 6 shows the results of a number of MAK column fractionations of the nucleic acids of seedlings harvested from the above plant growth experiments. In hypocotyls there seems, in general, to be a direct relationship between the overall growth response and the response of nucleic acid synthesis to treatment with these two chemicals. However, for GA at 10^{-6}M, a large increase in nucleic acid activity above control level did not mani-

fest itself in a comparable growth. This is interesting in light of the fact that in a plant variety that shows accelerated growth with GA it would be expected that the nucleic acid activity would be increased. Is it possible that here GA is acting on nucleic acids as it would in a plant in which it promotes growth but some other factor is arresting the translation of this promoted nucleic acid level into growth?

At the same time, in a plant whose hypocotyl and/or shoot growth is promoted by GA, the GA usually retards the growth of their roots. Table 6 shows that although GA has retarded nucleic acid activity in roots, this has not been reflected in a comparable reduction in root growth. Is it possible that the "translation factor" which was not adequate in the hypocotyls was so in the roots? This could allow near normal growth from sub-normal nucleic acid production.

Alternatively, GA is well known to induce the synthesis of hydrolytic enzymes in aleurone layers of barley seeds and in other plant tissues. GA could be inducing the production of ribonucleases in hypocotyls and inhibiting their production in roots, not an uncommon mode of action for plant hormones. This could result, in hypocotyls, in an increased specific activity without an increase in growth; while in roots the specific activity would be reduced but not necessarily the growth.

The interrelation between AMO-1618, growth, and nucleic acid synthesis seems to be fairly direct in hypo-

cotyls but in roots the AMO-1618 concentration that produces the greatest nucleic acid activity results in the least growth while AMO-1618 at 10^{-7}M, which does not change nucleic acid activity relative to controls, greatly increases growth.

When a retarding concentration of AMO-1618 (10^{-4}M) is combined with various concentrations of GA (10^{-8} and 10^{-4}) there seems to be a fairly direct relationship between nucleic acid activity and growth. In hypocotyls GA at 10^{-8}M plus AMO-1618 at 10^{-4}M acts like an augmented AMO-1618 at 10^{-4}M. GA (10^{-4}M) plus AMO-1618 (10^{-4}M) acts like a diminished AMO-1618 (10^{-7}M). A degree of competition between GA and AMO-1618 can be inferred from these results. In other words, a low GA concentration (10^{-8}M) effectively competes with the AMO-1618 to reduce the effective concentration of AMO-1618 slightly and therefore increasing nucleic acid activity and growth slightly. A high GA concentration (10^{-4}M) more effectively competes and reduces greatly the operative concentration of AMO-1618 and greatly increases nucleic acid activity and growth.

In roots, however, growth followed the effect of the AMO-1618 (10^{-4}M) with either GA (10^{-8}M) or (10^{-4}M) whereas nucleic acid activity seemed to show the reducing effect of the presence of GA as though the effects of the two chemicals was not as closely related as they were in hypocotyls.

DISCUSSION

The work reported in this thesis shows what appears to be a novel relationship between GA and AMO-1618. Ounsworth and Pillay (1969) have shown that the growth of shoots of light-grown soybeans is enhanced by GA and retarded by Alar. When, however, dark-grown soybean seedlings are treated with GA and AMO-1618 prior to the initiation of epicotyl growth, as was done in this thesis, the effect of GA on the growth is minimal while the effect of AMO-1618 is retarding at high concentrations and very stimulatory at low concentrations. Lang (1970) cautioned that plants giving such atypical responses to retardants "are no suitable material on which to use retardants for studying GA physiology".

This work, however, shows that an investigation of such atypical responses may afford even greater insight into the physiology of plant responses to these chemicals. Lang's stricture (idem) that an interaction between GA and a retardant "can be related causally only if the effect on growth can be completely overcome by applied GA" was probably only meant to apply to an overcoming of a retardation. It is now clear that GA can overcome a promotive effect of the growth retardant AMO-1618 and that this effect is somewhat different in roots and hypocotyls. In roots a retarding concentration of AMO-1618 (10^{-4}M) could not be overcome by GA but a promotive concentration (10^{-5}M) was abolished completely even by very low GA concentrations (10^{-10}M).

In hypocotyls GA was able to overcome both retarding (10^{-4}M) and promoting (10^{-5}M) concentrations of AMO-1618 in a manner that implies a close interaction between them. Increasing concentrations of GA acted as though the GA was effectively reducing the concentration of AMO-1618 until high GA concentration blocked completely the promotive AMO-1618 affect and the growth response became similar to the response to GA alone. It should be noted that roots could have a more finely tuned response to these chemicals and it could be that the use of fractional concentrations of AMO-1618 (between 10^{-5} and 10^{-4}) might evoke responses similar to those in hypocotyls.

The simple model of AMO-1618 action which suggests that it blocks gibberellin synthesis and thereby reduces the endogenous gibberellin level and thus retards growth cannot explain why added GA does not affect appreciably the growth of soybean hypocotyls and roots, and even retards them at higher concentrations. Neither does it explain how AMO-1618 can cause a promotion of growth which can be clearly altered by GA application. It may be that more subtle effects of AMO-1618 have been masked by the massive doses normally used to block GA synthesis completely (10^{-4}M and greater).

Lockhart (1962) has described kinetic studies which indicate that CCC and phosphon interact competitively with gibberellin on stem growth of light-grown pinto beans but not on root growth. He indicates that, although Lineweaver-Burk analysis is not possible because living systems cannot

be made free of endogenous growth factor, simple dose-response curves can supply considerable information. He says that "whenever a growth factor can completely eliminate the influence of a second factor in a properly controlled system, a competitive interaction is demonstrated."

The type of interaction exhibited in hypocotyls between GA and AMO-1618 at 10^{-4}M represents what would be expected if an allosteric enzyme were involved, where AMO-1618 is acting as the substrate and GA is acting as the competitive inhibitor. If this is the case then some allosteric enzyme like aspartate transcarbamylase which controls pyrimidine biosynthesis in E. coli could be worth investigation. The effect of AMO-1618 and GA on nucleotide pools might afford preliminary evidence of the involvement of such an enzyme. This is suggested by the work of Johri and Varner (1968) who found that nearest-neighbor analysis of RNA synthesized by GA-exposed pea nuclei showed a higher frequency of adenine- and guanine-containing pairs and a lower frequency of cytosine- and uracil-containing pairs. The attractiveness of this possibility is enhanced by the fact that AMO-1618, like the normal substrate for aspartate transcarbamylase, is a carbamate.

SUMMARY

A simplified, three-layered MAK column has been found to satisfactorily separate soybean nucleic acid extracts into their typical fractions. In addition the direct counting of aqueous solutions of ^{32}Pi-labelled nucleic acid fractions by the Cerenkov counting procedure of Haviland and Bieber (1970) has been found to give satisfactory, repeatable results.

Significant incorporation of ^{32}Pi into nucleic acid fractions from MAK columns begins 24 to 48 hours after imbibition starts. Roots appear to incorporate ^{32}Pi into nucleic acids much more efficiently than do hypocotyls or cotyledons.

High concentrations of AMO-1618 (10^{-4} and higher) progressively reduce ^{32}Pi incorporation in imbibing seeds and in 6 day old hypocotyls, but augment incorporation into roots. GA has little effect on incorporation by roots but seems to progressively reduce ^{32}Pi incorporation into hypocotyl nucleic acids. GA could not overcome the reduction in incorporation by a high concentration of AMO-1618 in roots but hypocotyls grown in a combination of GA and AMO-1618 showed a ^{32}Pi incorporation comparable to that obtained with a reduced AMO-1618 concentration alone.

AMO-1618 promoted the growth in length of both roots and hypocotyls at concentrations 10^{-5}M or lower, while higher concentrations significantly retarded both. GA is capable of overcoming the growth effect of a promotive

concentration of AMO-1618 in both parts of the plant; however, whereas it could not overcome an inhibitory AMO-1618 concentration in roots, it could in hypocotyls in a manner suggestive of the interaction of substrate and inhibitor on an allosteric enzyme. It is suggested that studies of an enzyme like aspartate transcarbamylase and of nucleotide pools in this system may prove fruitful.

Appendix

(Essentially as outlined by Osawa and Sibatani)

I Washing the Kieselguhr

1. Suspend 150 g Kieselguhr in 600 ml of 1 N NaOH overnight.
2. Filter on Buchner funnel and wash with 450 ml of 1 N NaOH.
3. Suspend in 300 ml of 1 N HCl overnight.
4. Filter and wash with 450 ml of 1 N HCl.
5. Wash with distilled water until no longer acid (approx. 1000 ml)
6. Suspend in distilled water.
7. Decant to remove fine particles--repeat once.

II Methylating the Albumin

(Essentially by the procedure of Mandell and Hershey)

1. Suspend 5 gm of bovine serum albumin (fraction V) in 500 ml of freshly opened absolute methanol.
2. Add 4.2 ml of concentrated HCl slowly with stirring.
3. Tightly seal and incubate in the dark at 37°C for 5 days. Shake often.
4. Quickly cool and centrifuge at 20,000 x g for 10 min.
5. Wash twice, or until yellow colour is gone, with cold methyl alcohol.
6. Wash twice with cold, newly opened, anhydrous ether.
7. Pour off supernatant and air dry residue while working it with a spatula to reduce it to a powder.

8. Store in vacuo over KOH.

NOTE For each new supply of methylated albumin a new salt gradient range must be established. This can be done by eluting a nucleic acid sample through the new MAK using a wide salt gradient (ex. 0.2M--2.0M). From the resulting profile a new, suitable gradient range can be determined.

III Preparation of Bentonite

(Essentially the method H. Fraenkel-Conrat et al, 1961)

1. Stir 10 g Bentonite in 200 ml of distilled water for 1 hour.
2. Centrifuge at 2500 rpm for 15 minutes.
3. Recentrifuge supernatant at 8500 rpm for 20 minutes.
4. Resuspend residue in 0.1 M Versene, pH 7, for 48 hrs. at 25°C.
5. Centrifuge at 2500 rpm for 15 minutes.
6. Centrifuge supernatant at 8500 rpm for 20 minutes.
7. Suspend residue in 0.01 M acetate buffer, pH 6.
8. Centrifuge at 8500 rpm.
9. Freeze-dry.
10. Make 40 mg/ml with 0.1 M acetate buffer, pH 5.0

IV Preparing the Column

Beaker I	Beaker II	Beaker III
1 g paper powder in 20 ml 0.1 M buffered saline	10 g Kieselguhr in 50 ml of 0.1M buffered saline	1 g Kieselguhr in 10 ml of 0.1 M buffered saline
	Boil (1 min)-- Cool	Boil (1 min)-- Cool
	Add 2.5 ml (slow & stir) 1% Methylated albumin	
Add to closed column (use inverted 10 ml pipette)	Gently layer on to paper	Gently layer on methylated kieselg. using pressure (2-3 psi)
Drip to pack then close	Drip to pack using pressure (2-3 psi)	
Carefully wash with 0.1 M	Wash with 200 ml of 0.1 M using pressure	Wash with 0.1 M
Drip to 1 cm of paper		Stop few mm from top

V Loading the Column

1. Dissolve 35 O.D.'s (1.75mg) in 40 ml of starting buffer.
2. Add to column at a rate of 1 ml/min. with pressure (2-3 psi)
3. Wash with 20 ml of starting buffer.
4. Wash with 10 ml of starting buffer.
5. Stop 1-2cm from top.

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